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A Video Bioinformatics Method to Quantify Cell Spreading and Its Application to Cells Treated with Rho-Associated Protein Kinase and Blebbistatin

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Chapter 8

A Video Bioinformatics Method to Quantify Cell Spreading and Its Application to Cells Treated with Rho-Associated Protein Kinase and Blebbistatin

Nikki Jo-Hao Weng, George Phandthong and Prue Talbot

Abstract Commercial software is available for performing video bioinformatics analysis on cultured cells. Such software is convenient and can often be used to create suitable protocols for quantitative analysis of video data with relatively little background in image processing. This chapter demonstrates that CL-Quant software, a commercial program produced by DRVision, can be used to automatically analyze cell spreading in time-lapse videos of human embryonic stem cells (hESC). Two cell spreading protocols were developed and tested. One was professionally created by engineers at DRVision and adapted to this project. The other was created by an undergraduate student with 1 month of experience using CL-Quant. Both protocols successfully segmented small spreading colonies of hESC, and, in general, were in good agreement with the ground truth which was measured using ImageJ. Overall the professional protocol performed better segmentation, while the user-generated protocol demonstrated that someone who had relatively little background with CL-Quant can successfully create protocols. The protocols were applied to hESC that had been treated with ROCK inhibitors or blebbistatin, which tend to cause rapid attachment and spreading of hESC colonies. All treatments enabled hESC to attach rapidly. Cells treated with the ROCK inhibitors or blebbistatin spread more than controls and often looked stressed. The use of the spreading analysis protocol can provide a very rapid method to evaluate the

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cytotoxicity of chemical treatment and reveal effects on the cytoskeleton of the cell. While hESC are presented in this chapter, other cell types could also be used in conjunction with the spreading protocol.

8.1 Introduction

Live cell imaging has been widely used in our laboratory for many years to study dynamic cell processes [9, 18, 20, 40–42] and has more recently been applied to toxicological problems [10, 12, 22, 23, 27, 32–36, 39]. Analysis of dynamic events, such as cell attachment, migration, division, and apoptosis, can provide mechanistic insight into normal cellular processes [28, 37] as well as how toxicants affect cells [2, 5, 23, 42]. Collection of video data has recently improved due to the introduction of commercial incubators with built-in microscopes and cameras for collecting time-lapse data during short- and long-term experiments [8, 30, 37].

After videos are collected, it is important to extract quantitative data from them. A challenging but important issue until recently has been how to analyze large complex data sets that are produced during live cell imaging. When video data analyses are done manually by humans, many hours of personnel time are usually required to complete a project, and manual analysis by humans is subject to variation in interpretation and error. Video bioinformatics software can be used to speed the analysis of large data sets collected during video imaging of cells and can also improve the accuracy and repeatability of analyses [37]. Video bioinformatics, which involves the use of computer software to mine specific data from video images, is concerned with the automated processing, analysis, understanding, visualization, and knowledge extracted from microscopic videos. Several free video bioinformatics software packages are available online such as ImageJ and Gradientech Tracking Tool (<http://gradientech.se/products/gradientech-tracking-tool/>). Also, some advanced video bioinformatics software packages, such as CL-Quant, Amira, and Cell IQ, are now commercially available and can be used to generate customized protocols or libraries to analyze video data and determine quantitatively how cells behave during experimental conditions.

This chapter presents a new application of CL-Quant software to automatically analyze cell spreading in time-lapse videos of human embryonic stem cells (hESC) (WiCell, Madison, WI). While hESC are presented in this chapter, other cell types could also be used in conjunction with these protocols.



8.2 Instrumentation Used to Collect Live Cell Video Data

8.2.1 *BioStation IM*

Data were collected with a BioStation IM. The BioStation IM or its newer version the IM-Q, manufactured by Nikon, is a bench top instrument that houses a motorized inverted microscope, an incubator with a built-in high sensitivity cooled CCD camera, and software for controlling exposures, objectives, and the type of imaging (e.g., phase contrast or fluorescence). The components of this instrument are fully integrated and easy to set up. In a BioStation IM, cells are easily maintained at a constant temperature (37 °C) and relative humidity (85 %) in a 5 % CO₂ atmosphere. The BioStation IM enables time-lapse data to be collected reliably over hours or days without focus or image drift. In time-lapse experiments, images can be collected as quickly as 12 frames/s. In a BioStation IM, imaging can be also performed in the X-, Y-, and Z-direction. The unit comes with a well-designed software package and a GUI for controlling the instrument and all experimental parameters.

The BioStation IM is available in two models, the BioStation II and BioStation II-P, optimized for either glass bottom or plastic bottom culture dishes, respectively, and the magnification range is different in the two models. Both models accommodate 35 and 60 mm culture dishes. A four-chambered culture dish, the Hi-Q4 sold by Nikon, can be used for examining four different conditions of culture in the same experiment.

8.3 Software Used for Video Bioinformatics Analysis of Stem Cell Morphology and Dynamics

8.3.1 *CL-Quant*

CL-Quant (DRVision, Seattle, WA) provides tools for developing protocols for recognition and quantitative analysis of images and video data [1]. The software is easy to learn and does not require an extensive knowledge of image processing. CL-Quant can be used to detect, segment, measure, analyze, and discover cellular behaviors in video data. It can be used with both phase contrast and fluorescent images. Several basic protocols for cell counting, cell proliferation, wound healing, and cell migration have been created by DRVision engineers and can be obtained when purchasing CL-Quant software. Protocols can be created by DRVision at a user's request, or users can create their own protocols. Later in this chapter, we will describe how to create a protocol in CL-Quant, show the difference between a professional protocol and user-generated protocol, and also show an example in which CL-Quant was used to measure hESC spreading in experimental time-lapse videos.



8.3.2 ImageJ

ImageJ is a public domain Java-based image-processing program, which was developed at the National Institutes of Health. ImageJ was designed with an open architecture that provides extensibility via plug-ins and recordable macros. A number of tutorials are available on YouTube and are helpful for beginners learning to use this software. ImageJ is compatible with major operating systems (Linux, Mac OS X, and Windows), works with 8-bit color and grayscale, 16-bit integer, and 32-bit floating point images. It is able to read many image formats, and it supports **time**- or z-stacks. There are numerous plug-ins that can be added to ImageJ to help solve many imaging processing and analysis problems. ImageJ is able to perform numerous standard image-processing operations that may be useful in labs dealing with image analysis and video bioinformatics. For example, researchers have used ImageJ to quantify bands in western blots and also to quantify the fluorescent intensity on the images. One of the advantages of using ImageJ is that it enables rapid conversion of images to different formats. For example, ImageJ can convert tif images to avi, it can create 3D images from z-stacks with 360° rotation, and it can be used to obtain ground truth information when setting up a new video bioinformatics protocol.

8.4 Protocols for Cell Attachment and Spreading

In toxicological assays using stem cells, endpoints of interest often occur days or sometimes a month after the experiment begins [27, 37]. There has been interest in shortening such assays, often by using molecular biomarkers to obtain endpoints more rapidly [6]. In the mouse embryonic stem cells test, endpoints can now be obtained in 7 days using biomarkers for heart development. While this significantly reduces the time to reach an endpoint, it is still a relatively long time to obtain data on cytotoxicity.

Pluripotent hESC model the epiblast stage of development [31] and are accordingly a valuable resource for examining the potential effects of chemicals at an early stage of human prenatal development [38]. We are developing video assays to evaluate cellular processes in hESC in short-term cultures, and we then use these assays to identify chemicals that are cytotoxic to young embryos. One hESC-based assay involves evaluation of cell spreading, a dynamic process dependent on the cytoskeleton. When a treatment alters the cytoskeleton or its associated proteins, cells are not able to attach and spread normally. We have used two video bioinformatics protocols to analyze these parameters during 4 h of in vitro culture. At the beginning of cell plating, hESC are round and unattached. Usually, hESC attach to their substrate and begin spreading within 1 h of plating. As cells attach and start spreading, their area increases, and this can be measured in time-lapse images using video bioinformatics tools, thereby providing a rapid method to evaluate a process



dependent on the cytoskeleton. Two parameters can be derived from the time-lapse data: rate of cell spreading (slope) and fold increase in cell area. These two parameters were compared in control and treated groups using the linear regression and 2-way ANOVA analysis (GraphPad Prism, San Diego).

We will compare two protocols for measuring cell spreading in this chapter. Both protocols are performed using CL-Quant software. One, which we term a professional protocol, was created by DRVision engineers for quantifying cell proliferation. Even though the professional protocol was not created specifically for hESC spreading, we were able to use the segmentation portion of the protocol for measuring spreading (area) of hESC during attachment to Matrigel. In addition, we created our own protocol using CL-Quant for analyzing the area of hESC colonies during attachment and spreading. Our method, which we refer to as the user-generated protocol, was created by an undergraduate student with a basic background in biology and 1 month of experience with CL-Quant software.

hESC were cultured using methods described in detail previously [29]. To create time-lapse videos for this project, small colonies of hESC were incubated in mTeSR medium at 37 °C and 5 % CO₂ in a BioStation IM for 4 h. Frames were captured every minute from 4–5 different fields. When applying either the professional or user-generated protocol, the first step was to segment the image so as to select mainly hESC. During segmentation, we used the DRVision's soft matching procedure, which allowed us to identify the objects of interest. The second step was to remove noise and small particles/debris that were masked during segmentation. In the third step, the area of all cells in a field was measured in pixels. The protocol was applied to all images in time-lapse videos to obtain the area occupied by hESC when plated on Matrigel and incubated for 4 h. Because the survival efficiency is low for single cells [43], hESC were plated as small colonies, which normally attach, spread, and survive well. Figure 8.1a shows phase contrast images of several small hESC colonies plated on Matrigel at different times over 4 h. In the first frame, the cells were unattached, as indicated by the bright halo around the periphery of some cells. During 4 h of incubation, the cells attached to the Matrigel and spread out. By the last frame, all cells in the field have started to spread. Figure 8.1a also shows the same images after segmentation, enhancement, and masking using the professional protocol supplied by DRVision. Comparison of the phase contrast and segmented sequences shows that the masks fit each cell well.

To determine if the measurement data obtained from the professional CL-Quant protocol were accurate, ground truth was obtained by tracing each hESC in all of the video images using the freehand selection tool in ImageJ, and then measuring the pixel area for each frame. The ground truth (dotted line) and CL-Quant derived area were very similar for hESC grown in control conditions (mTeSR medium) (Fig. 8.1b). For most videos, CL-Quant slightly overestimated the area of the cells due to difficulty in fitting a perfect mask to each cell.

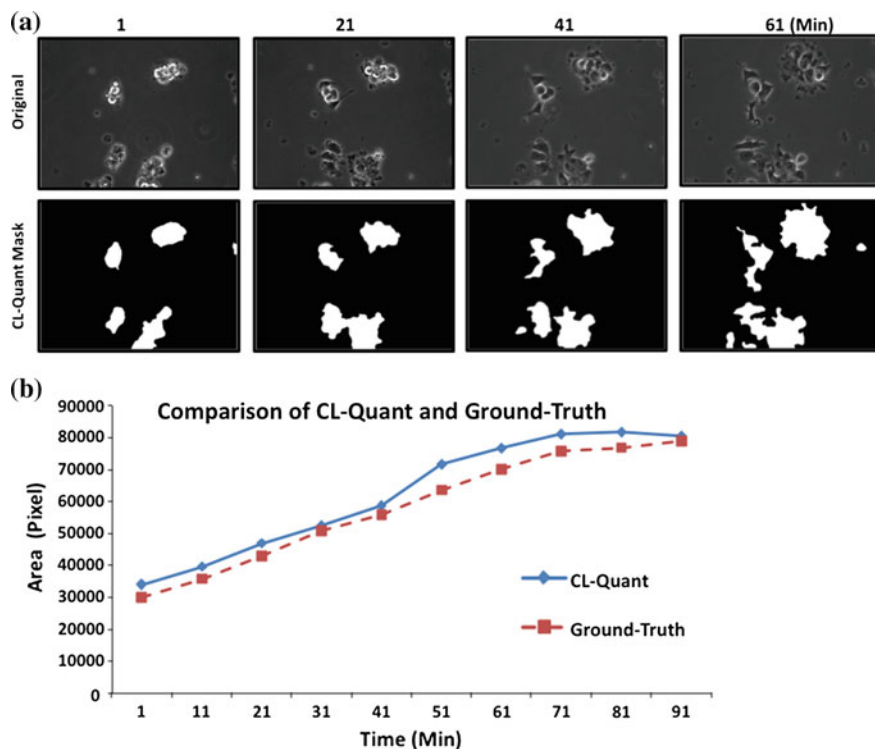


Fig. 8.1 Comparison of CL-Quant segmentation protocol and ground truth. hESC were plated in mTeSR medium in a 35 mm dish and incubated in a BioStation IM for 4 h. **a** Phase contrast images modified with ImageJ to remove text labels and the same images with masks applied using the professional CL-Quant protocol. **b** Graph showing cell area (spreading) in pixels for CL-Quant derived data and the ground truth. The areas obtained from the two methods were in good agreement

8.5 Application of Video Bioinformatics Protocols to hESC Cell Spreading in the Presence of Rock Inhibitors and Blebbistatin

In 2007, ROCK inhibitor (Y27632) was shown to increase the efficiency of hESC survival in culture [43]. However, ROCK is involved in numerous signaling pathways, and therefore may affect many cell properties [25]. ROCK inhibitors decrease non-muscle myosin II activity, and this decrease helps attachment of hESC. However, when single hESC are plated with ROCK inhibitor, they do not adhere to each other due to downregulation of e-cadherin [19]. In addition, hESC treated with Y27632, a potent ROCK inhibitor, appeared stressed and less healthy than untreated controls [3]. Finally, the use of ROCK inhibitor (Y27632) in a toxicological study with methyl mercury decreased the IC_{50} [11]. Blebbistatin, an



inhibitor of myosin II which is downstream of ROCK, can also be used to increase attachment of hESC and thereby improve plating efficiency [19] and may also alter cell morphology [21].

In this study, time-lapse data were collected on hESC treated with different ROCK inhibitors or blebbistatin, and two CL-Quant protocols were used to quantitatively compare spreading of treated cells to controls. H9 hESC were seeded on Hi-Q4 dishes coated with Matrigel and incubated using different treatment conditions. Cells were then placed in a BioStation IM and cultured as described above for 4 h during which time images were taken at three or four different fields in each treatment/control group at 1 min intervals.

The protocols described in Sect. 1.4 for quantifying hESC area were used to compare spreading of cells subjected to different ROCK inhibitors (Y27632, H1152) or to blebbistatin. First, the written data that was stamped on each image by the BioStation software was removed using the remove outlier's feature in ImageJ. The professional CL-Quant protocol was applied to the resulting time-lapse videos. Examples of phase contrast and masked images are shown in Fig. 8.2 for treatment with the two ROCK inhibitors and blebbistatin. Cells in each group were masked accurately by the segmentation protocol, and even thin surface cell projections were masked with reasonable accuracy. The measurement protocol was then applied to each frame to determine the area (pixels) of the masked cells. To establish the accuracy of this protocol, the ground truth for control and treated groups was determined using ImageJ in two separate experiments (Fig. 8.3a, b). Each point in Fig. 8.3 is the mean of 3 or 4 videos. As shown in Fig. 8.3, the ground truth and the CL-Quant derived data were in good agreement for all groups in both experiments.

In the first experiment, the fold increase in spread area was elevated by Y27632 and blebbistatin relative to the control ($p < 0.0001$ for Y27632 and $p < 0.05$ for blebbistatin 2-way ANOVA, Graphpad Prism), while H1152 was not significantly different than the control ($p > 0.05$). The rate of spreading, as determined by the slope for each group, was greater in the three treated groups than in the control; however, only the slope for Y27632 was significantly different than the control ($p < 0.0001$) (slopes = 0.124 control; 0.135 H1152; 0.142 blebbistatin; 0.245 Y27632). In this experiment, the Y27632 group was distinct from all other groups in both its rate of spreading and fold increase in spread area. The morphology of the control cells was normal; cells had smooth surfaces with relatively few projections. In contrast, all treated cells had irregular shapes and more projections than the controls (Fig. 8.3a).

In the second experiment, the three treated groups spread faster and more extensively than the control group. All three treated groups were significantly different than the control with respect to fold change in spread area (by 2-way ANOVA $p < 0.05$ for Y27632 and H1152; $p < 0.0001$ for blebbistatin). In contrast to the first experiment, the Y27632 group was similar to the other two treatments (Fig. 8.3d). The rate of spreading was greater in the three treated groups than in the control (slope = 0.084 control; 0.117 H1152; 0.150 blebbistatin; 0.136 Y27632), and both Y27632 ($p < 0.01$) and blebbistatin ($p < 0.001$) were significantly different than the control. As seen in the first experiment, all treated cells were

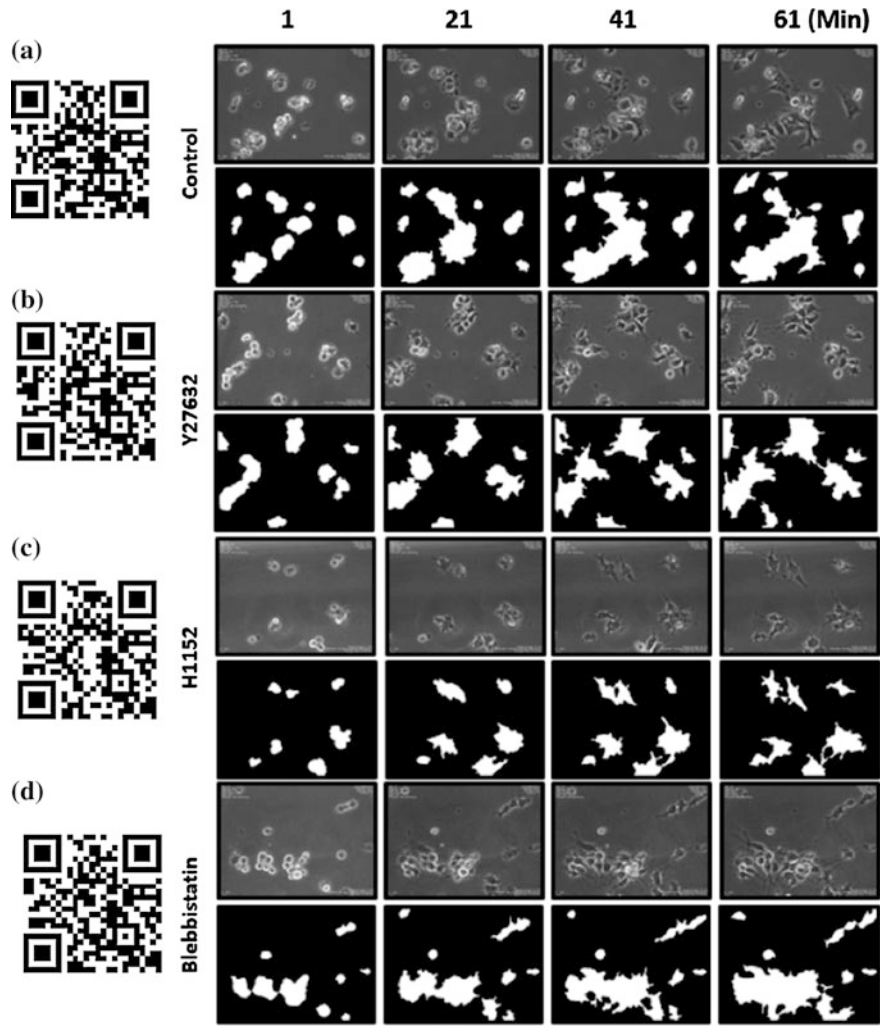


Fig. 8.2 Cell area (spreading) was successfully masked by the professional CL-Quant protocol in different experimental conditions. hESC were treated with ROCK inhibitors (Y27632 and H1152) or blebbistatin, incubated in a BioStation IM for 4 h, and imaged at 1 min intervals. Phase contrast images and the corresponding masked images are shown for hESC treated with: **a** control medium, **b** Y27632, **c** H1152, and **d** blebbistatin

morphologically distinct from the controls. Treated cells appeared attenuated and had long thin projections extending from their surfaces, while control cells were compact and had smooth surfaces (Fig. 8.3c). It is possible that CL-Quant underestimated the area of the Y27632 inhibitor treated cells in the second experiment due to the attenuation of the surface projections, which were more extensive than in the first experiment and were difficult to mask accurately.

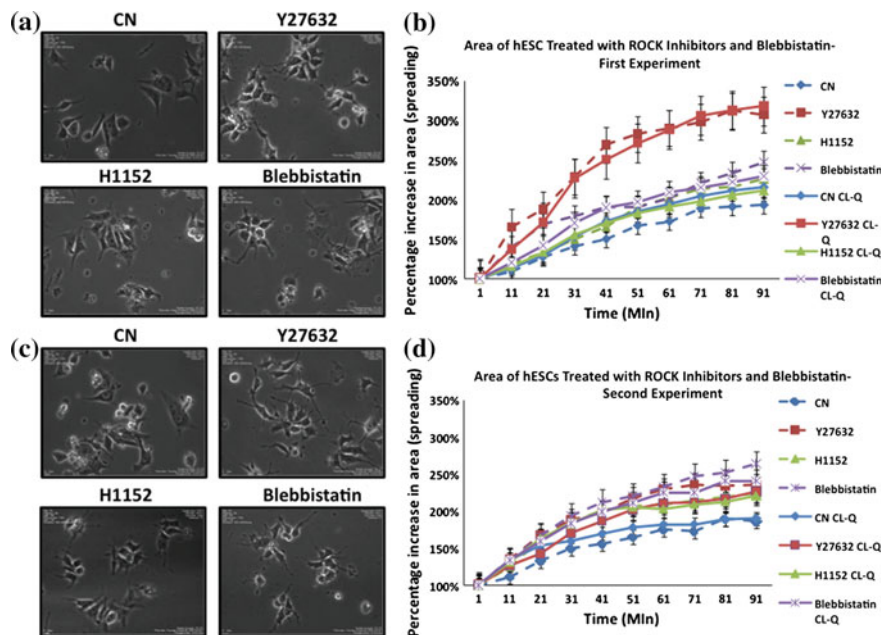


Fig. 8.3 The morphology and spreading of hESC was affected by treatment with ROCK inhibitors (Y27632 and H1152) and blebbistatin in two experiments. Spreading was measured using the professional CL-Quant protocol. **a** Phase contrast images from the first experiment showed that treated cells were morphologically different than the control. **b** The rate of spreading and the fold increase in spread area was greater in Y27632 and blebbistatin treated cells than in controls. **c** Phase contrast images of control and treated cells in the second experiment showed morphological changes in the treated groups. **d** The fold increase in spread area was greater in the treated cells than in the controls in the second experiment; however, the effect of Y27632 was not as great as previously seen. Data in (b) and (d) are plotted as a percentage of the area in the first frame. Each point is the mean \pm the SEM

Videos showing the effect of ROCK inhibitors and blebbistatin on hESC spreading can be viewed by scanning the bar code.

8.6 Comparison of Professional and User-Generated Protocols

We also compared the professional and user-generated protocols to each other. The cells in both the control and treated groups were well masked by the professional protocol, and the mask included the thin surface projections characteristic of the treated group (Fig. 8.4a). To validate the quantitative data obtained with the professional protocol, ground truth was determined using ImageJ (Fig. 8.4b). Both the

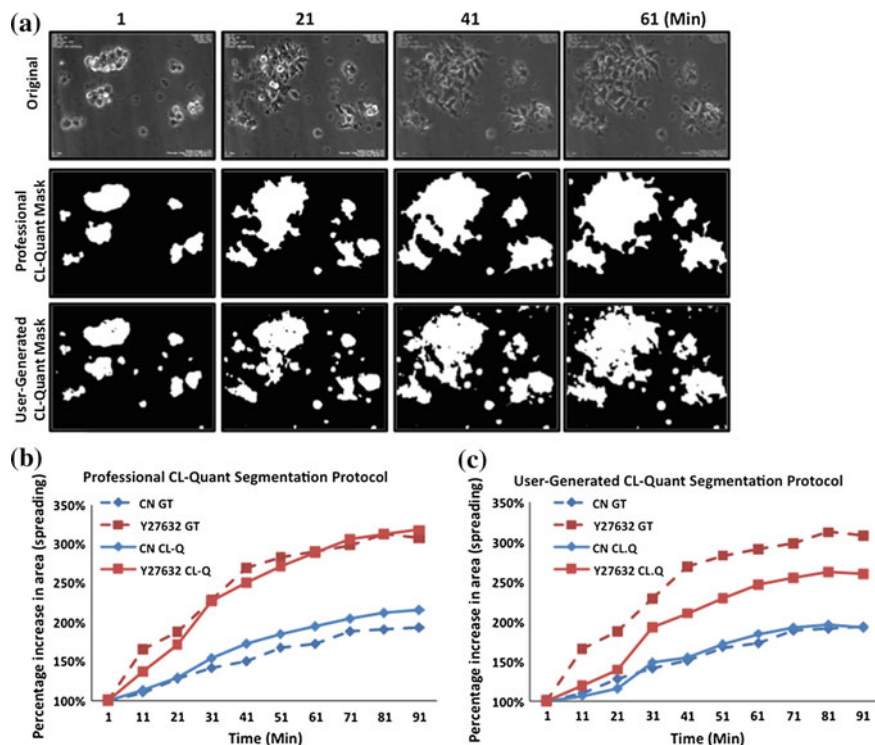


Fig. 8.4 Comparison of the professional and user-generated cell spreading protocols. **a** Phase contrast micrographs of hESC treated with Y27632 and the corresponding masks created with the professional and user-generated protocols. **b** Comparison of ground truth to area (spreading) data obtained with the professional protocol in control and treated groups. **c** Comparison of ground truth to area (spreading) data obtained with the user-generated protocol in control and treated groups

control and treated groups were in good agreement with the ground truth (Fig. 8.4b).

An advantage CL-Quant is that users can generate their own protocols without a programming background. Our user-generated protocol, which was created by an undergraduate student with 1 month of experience using CL-Quant, was applied to the control and treated groups. The resulting masks did not cover the surface projections of the treated group as well as the professional protocol; however, the user-generated protocol did include single cells, some of which were filtered out by the professional protocol (Fig. 8.4a). The user-generated protocol did not filter out the small debris as well as the professional protocol (Fig. 8.4a). The data obtained with the user-generated protocol were close to ground truth for the control group, but not for the treated group (Fig. 8.4c). Phase contrast images showed that the cells treated with Y27632 had many more attenuated surface projections than the control cells (Fig. 8.5a, d). Our user-generated protocol and the professional protocol were

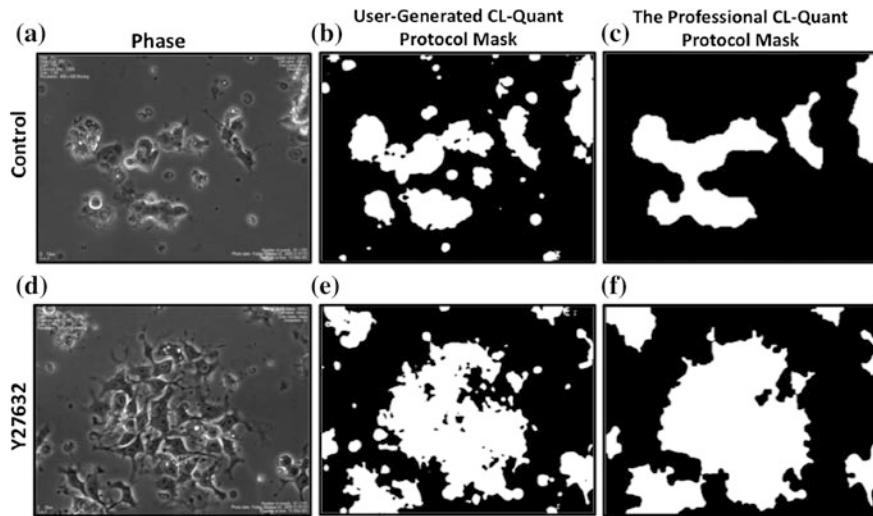


Fig. 8.5 Differences in cell morphology showing why treated hESC are more difficult to segment than control cells. **a** Phase contrast image of hESC colonies taken at 60 min of incubation. Segmentation of the image in “(a)” created with the user-generated protocol (**b**) and the professional protocol (**c**). **d** Phase contrast image of hESC colonies treated with Y27632 for 60 min. The cells have many thin surface projections not present on controls. Segmentation of the image in “(c)” with the user-generated protocol (**e**) and the professional protocol (**f**)

able to mask the control cells well (Fig. 8.5b, c). However, the user-generated protocol was not able to mask the thin projections on treated cells as well as the professional protocol (Fig. 8.5e, f). Neither protocol recognized gaps between cells in the treated group. Overall, the professional protocol was more similar to the ground truth than the user-generated protocol in this experiment; however, with more experience, the user could improve the protocol to include surface projections more accurately.

8.7 Discussion

In this chapter, we introduced a video bioinformatics protocol to quantify cell spreading in time-lapse videos using CL-Quant image analysis software, and we validated it against ground truth. A professionally developed version of the protocol was then compared to a protocol developed by a novice user of CL-Quant. We also applied this protocol to hESC treated with blebbistatin and ROCK inhibitors, which are commonly used during in vitro passaging of hESC [43].

Most evaluations of cells in culture have involved processes such as cell division, confluency and motility, but not spreading. Cell attachment and spreading depend on the interaction between cells and the extracellular matrix to which they



attach. When cells are plated on a substrate, they first attach, then flatten and spread. At the molecular level, spreading depends on the interaction of membrane-based integrins with their extracellular matrix and the engagement of the cytoskeleton, which initiates a complex cascade of signaling events [7]. This in turn leads to the morphological changes observed during spreading and enables cultured cells to flatten out and migrate to form colonies [24, 44].

Changes in cell behavior that depend on the cytoskeleton often indicate that cell health is being compromised by environmental conditions [2, 27]. Because spreading depends on the cytoskeleton, it can be used to evaluate the effect of chemical treatments on cytoskeletal health. Cell spreading is a particularly attractive endpoint in toxicological studies as it occurs soon after plating and does not require days or weeks of treatment to observe. Many types of cells, such as fibroblasts can attach and spread in 10–15 min. hESC require about 1–2 h to spread, and therefore can be plated and data collected in 4 h or less time depending on the experimental design.

The application of the spreading protocol to hESC enables chemical treatments to be studied using cells that model a very early stage of postimplantation development. hESC are derived by isolating and culturing the inner cell mass from human blastocysts. As these cells adapt to culture, they take on the characteristics of epiblast cells which are found in young post-implantation embryos [31]. Embryonic cells are often more sensitive to environmental chemicals than differentiated adult cells, and it has been argued that risk assessment of environmental chemicals should be based on their effects on embryonic cells, as these represent the most vulnerable stage of the life cycle [13]. The sensitivity of hESC to environmental toxicants may be due to mitochondrial priming which occurs in hESC and makes them more prone to apoptosis than their differentiated counterparts [26].

A major advantage of the cell spreading assay is that it requires relatively little time to perform. A complete spreading assay can be done in as little as 4 h, while other endpoints such as cell division and differentiation require days or weeks to evaluate. The rapidity of the spreading assay makes it valuable in basic research on the cytoskeleton or in toxicological studies involving drugs or environmental chemicals. Moreover, this tool could be used in the future as a quality control check when evaluating stem cell health for clinical applications.

The cell spreading assay introduced in this chapter provides a rapid method for evaluating the cytoskeleton and assessing the quality of cells. Using bioinformatics tools to analyze the video data significantly reduces the time for data analysis [14–17]. If an experiment is done for 4 h with 1 min intervals between frames, 240 frames would be collected for each video by the end of the experiment. Each group could have 4–10 different videos. Before we used bioinformatics tools to analyze our data, cell spreading was analyzed by measuring cell area manually. ImageJ was used to calculate cell area for each frame. The total time for cell spreading analysis for each video was about 24 h. The CL-Quant bioinformatics protocol, which we introduced in this chapter, greatly reduces this time. In general, it takes about 30–60 min to create a protocol for a specific purpose. Once the protocol is created, it takes 5 min to run the protocol using CL-Quant. The protocol can be batch run on



multiple videos without requiring users to tie up valuable time performing analysis of spreading.

Our data show that the professionally developed protocol performed better than the one developed by the novice user. However, the novice was able to rapidly learn to use CL-Quant software, and he obtained accurate control data as shown by comparison to the ground truth. It was clear that the novice had difficulty masking the fine projections on the hESC surfaces, but with additional training, he would likely be able to create a better segmentation protocol and achieve more accurate masking of the treated group data.

We also examined the effect of ROCK inhibitors and blebbistatin on hESC spreading. The ROCK inhibitors and blebbistatin improved cell attachment and spreading, as reported by others [19, 43]. However, those hESC treated with ROCK inhibitors or blebbistatin appeared stressed, had thin attenuated projections off their surfaces, and did not seem as healthy as control cells. ROCK inhibitor or blebbistatin are often used to improve cell survival, especially when plating single cells. ROCK inhibitor and blebbistatin allow cells to be accurately counted and plated. Cell survival is also improved by the efficient attachment observed when these ROCK inhibitors and blebbistatin are used. Cells are stressed during nucleofection, so ROCK inhibitors are often used to improve cell survival when nucleofected cells are ready to plate. ROCK inhibitors and blebbistatin inhibit ROCK protein and downregulate myosin II activity which accelerates cell attachment and cell spreading [43]. Our analysis shows that both ROCK inhibitors and blebbistatin alter the morphology of spreading cells. Moreover, Y27632 and blebbistatin significantly increased the rate of spreading and the fold change in spread area when compared to untreated controls, which may be a factor in why they are more commonly used than H1152. While the full significance of the above changes in cell morphology and behavior are not yet known, these inhibitors do either indirectly or directly decrease myosin II activity, which may lead to the stressed appearance of the treated cells. It has not yet been established why decreasing myosin II activity leads to more rapid and extensive spreading of hESC.

Use of ROCK inhibitors is not recommended in toxicological applications of hESC as they can alter IC_{50} s [11]. A spectrophotometer method has been established to determine cell number when hESC are in small colonies [4]. This method enables an accurate number of cells to be plated without the use of ROCK inhibitors and may generally be applicable to hESC culture and would avoid the morphological stress observed in ROCK inhibitor treated cells.

The protocols reported in this chapter can be used to quantify two parameters of cell spreading, rate and fold change. The use of spreading as an assay for cytoskeletal responses and cell health is attractive as it takes relatively little time to collect data and analyze data with the application of video bioinformatics tools. In the future, the segmentation and filtering aspects of these protocols may be improved to gather more accurate data on the challenging cell surface projections, but these protocols in their current form can be reliably applied to spreading of hESC colonies. In the future, improvements in software could include more advanced



preprocessing tools, tools for analyzing 3-dimensional data, and tools for analyzing different cell morphologies.

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